Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis (for FFPE Samples)

Protocol

Version 1.0, August 2007

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In This Guide...

This guide describes Agilent's recommended operational procedures to analyze DNA copy number variations using Agilent 60-mer oligonucleotide microarrays for array-based comparative genomic hybridization (aCGH) analysis. This protocol is specifically developed and optimized to process DNA samples derived from formalin-fixed paraffin-embedded (FFPE) tissues.

1 Before You Begin

This chapter contains information (such as procedural notes, safety information, required reagents and equipment) that you should read and understand before you start an experiment.

2 DNA Isolation

This chapter describes the method to isolate genomic DNA (gDNA) from FFPE samples prior to labeling.

3 DNA Labeling

This chapter describes the steps to chemically label the gDNA samples with fluorescent dyes through ULS technology.

4 Microarray Processing and Feature Extraction

This chapter describes the steps to hybridize, wash and scan Agilent CGH microarrays and to extract data using the Agilent Feature Extraction Software.

5 Reference

This chapter contains reference information related to the protocol.

Contents

1 Before You Begin 7

Procedural Notes 8
Safety Notes 9
Agilent Oligo CGH Microarray Kit Contents 10
Required Equipment 12
Required Reagents 14
Required Hardware and Software 15

2 DNA Isolation 17

Step 1. Paraffin Removal 19
Step 2. Proteinase K Treatment 20
Step 3. gDNA Extraction 21
Step 4. gDNA Quantitation and Quality Analysis 23
Supplemental Procedure - To isolate gDNA from frozen tissues 25

3 DNA Labeling 27

Step 1. Preparation of gDNA Before Labeling 28
Step 2. Heat Fragmentation 29
Step 3. ULS Labeling 29
Step 4. Free Dye Removal 32

4 Microarray Processing and Feature Extraction 35

Step 1. Preparation of Labeled Genomic DNA for Hybridization 36
Step 2. Microarray Hybridization 41
Step 3. Wash Preparation 43
Step 4. Microarray Washing 46
Step 5. Microarray Scanning using Agilent or GenePix Scanner 51
Step 6. Data Extraction using Feature Extraction Software 53

5 Reference 61

Supporting User Guides 62

Agilent Microarray Layout and Orientation 63
Agilent Information Assets Access Agreement 66

1 Before You Begin

Procedural Notes 8
Safety Notes 9
Agilent Oligo CGH Microarray Kit Contents 10
Required Equipment 12
Required Reagents 14
Required Hardware and Software 15

Make sure you read and understand the information in this chapter and have the necessary equipment and reagents listed before you start an experiment.

Procedural Notes

- Follow the procedure described in this document to isolate gDNA from FFPE samples to increase the likelihood of a successful experiment.
- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves, and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- Maintain a clean work area.
- Do not vortex stock solutions and reactions containing gDNA. Instead, mix gDNA samples by gently tapping the tube with your finger.
- Avoid repeated freeze-thaw cycles of stock and diluted gDNA solutions.
- When preparing frozen reagent stock solutions for use:
  1. Thaw the aliquot as quickly as possible without heating above room temperature.
  2. Mix briefly on a vortex mixer, then spin in a microcentrifuge for 5 to 10 seconds to drive the contents off the walls and lid.
  3. Store on ice or in a cold block until use.
- In general, follow Biosafety Level 1 (BL1) safety rules.

Safety Notes

CAUTION Wear appropriate personal protective equipment (PPE) when working in the laboratory.

WARNING

- **ULS-Cy3 and ULS-Cy5 are considered hazardous by the OSHA Hazard Communication Standard(29 CFR 1910.1200). Contains material which causes damage to the following organs: kidneys, liver, cardiovascular system, respiratory tract, skin, eye lens or cornea, stomach. May be harmful if swallowed. Avoid contact with eyes, skin and clothing.**
- **Agilent-CGHblock may be harmful if swallowed. Avoid contact with eyes, skin and clothing.**
- **2X CGH and 2X Hi-RPM Hybridization Buffers are considered hazardous by the OSHA Hazard Communication Standard (29 CFR 1910.1200). Contains material which causes damage to the following organs: skin, central nervous system. May be harmful if swallowed. Avoid contact with eyes, skin and clothing.**
- **Agilent Stabilization and Drying Solution is considered hazardous by the OSHA Hazard Communication Standard (29 CFR 1910.1200). Flammable liquid and vapor. Keep away from heat, sparks and flame. Keep container closed. Use only with adequate ventilation. This solution contains material which causes damage to the following organs: kidneys, liver, cardiovascular system, upper respiratory tract, skin, central nervous system (CNS), eye, lens or cornea.**

Agilent Oligo CGH Microarray Kit Contents

Store entire kit at room temperature. After breaking the microarray foil pouch, store the microarray slides at room temperature (in the dark) under a vacuum desiccator or N_2 purge box. Do not store microarray slides in open air after breaking foil.

Catalog CGH Microarray Kits

- Five or ten microarrays printed on five 1-inch × 3-inch glass slides
- CD containing microarray design files in various file formats

Available designs include:

Human Genome CGH Microarray Kit 244A (p/n G4411B) (1x244K)

Human Genome CGH Microarray Kit 105A (p/n G4412A) (2x105K)

Mouse Genome CGH Microarray Kit 244A (p/n G4415A) (1x244K)

Mouse Genome CGH Microarray Kit 105A (p/n G4416A) (2x105K)

Rat Genome CGH Microarray Kit 244A (p/n G4435A) (1x244K)

Rat Genome CGH Microarray Kit 105A (p/n G4436A) (2x105K)

Unrestricted High-Definition CGH (HD-CGH) Microarray Kits

- One or four microarray(s) printed on each 1-inch x 3-inch glass slide
- Number of microarrays varies per kit and per order
- CD containing microarray design files in various file formats

Available designs include:

Human Genome CGH Microarray 244A-Supplemental (p/n G4423B, AMADID 016266)

Human Genome CGH Microarray 4x44K (p/n G4426B, AMADID 014950)

Mouse Genome CGH Microarray, 4x44K (p/n G4426B, AMADID 015028)

Custom High-Definition CGH (HD-CGH) Microarray Kits

- One, two, four or eight microarray(s) printed on each 1-inch × 3-inch glass slide
- Number of microarrays varies per kit and per order

Available formats include:

Custom HD-CGH Microarray, 1x244K (p/n G4423A)

Custom HD-CGH Microarray, 2x105K (p/n G4425A)

Custom HD-CGH Microarray, 4x44K (p/n G4426A)

Custom HD-CGH Microarray, 8x15K (p/n G4427A)

Required Equipment

Description	Company and part no.
Agilent Microarray Scanner Bundle	Agilent p/n G2565BA
Hybridization Chamber, stainless	Agilent p/n G2534A
Hybridization Chamber gasket slides, 5-pack (alternative packaging sizes are available) 1x244K microarrays or 2x105K microarrays or 4x44K microarrays or 8x15K microarrays	 Agilent p/n G2534-60003 or Agilent p/n G2534-60002 or Agilent p/n G2534-60011 or Agilent p/n G2534-60014
Hybridization oven; temperature set at 65°C	Agilent p/n G2545A
Hybridization oven rotator for Agilent Microarray Hybridization Chambers	Agilent p/n G2530-60029
UV-Transilluminator with SYBR photographic filter	Alpha Innotech p/n Alphalmager 2000 or equivalent
Nuclease-free 1.5 mL microfuge tubes (sustainable at 95°C)	Ambion p/n AM12400 or equivalent
Magnetic stir bar (×2 or ×4)*	Corning p/n 401435 or equivalent
Magnetic stir plate (×1 or ×3)*	Corning p/n 6795-410 or equivalent
Magnetic stir plate with heating element	Corning p/n 6795-420 or equivalent
Microcentrifuge	Eppendorf p/n 5417R or equivalent
PCR machine with heated lid	Eppendorf p/n 950000015 or equivalent
Nuclease-free 0.2 mL PCR tubes, thin-walled	Eppendorf p/n 951010006 or equivalent
Thermomixer	Eppendorf p/n 022670000 or equivalent
E-Gel Opener†	Invitrogen p/n G5300-01
E-Gel PowerBase v.4†	Invitrogen p/n G6200-04
UV-VIS spectrophotometer	NanoDrop p/n ND-1000 or equivalent

Description (continued)	Company and part no.
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
1.5 L glass dish	Pyrex p/n 213-R or equivalent
250 mL capacity slide-staining dish, with slide rack (×3 or ×5)*	Wheaton p/n 900200 or equivalent
Circulating water baths or heat blocks set to 37°C, 70°, 90° and 95°C	
Ice bucket	
Powder-free gloves	
Speed-vac	
Sterile, nuclease-free aerosol barrier pipette tips	
Timer	
Vacuum dessicator or N_2 purge box for slide storage	
Vortex mixer	

* The number varies depending on if wash procedure A or B is selected.

† Optional when Invitrogen E-gels are used.

Required Reagents

Description	Company and part no.
Oligo aCGH Labeling Kit for FFPE Samples	Agilent p/n 5190-0419
Genomic DNA Purification Module (includes a pack of 10 Agilent KREApure columns)	Agilent p/n 5190-0418
Agilent Oligo aCGH Hybridization Kit	Agilent p/n 5188-5220 (25) or p/n 5188-5380 (100)
Agilent Oligo aCGH Wash Buffer 1 and 2 set *or* Agilent Oligo aCGH Wash Buffer 1 (4 L) Agilent Oligo aCGH Wash Buffer 2 (4 L)	Agilent p/n 5188-5226 Agilent p/n 5188-5221 Agilent p/n 5188-5222
Stabilization and Drying Solution, 500 mL*	Agilent p/n 5185-5979
Phosphate Buffered Saline pH 7.4 (PBS)	Amresco p/n E504-500ML
TrackIt 1 Kb DNA Ladder	Invitrogen p/n 10488-072
DNase/RNase-free distilled water	Invitrogen p/n 10977-015
Human Cot-1 DNA *or* Mouse Cot-1 DNA *or* Rat Hybloc	Invitrogen p/n 15279-011 Invitrogen p/n 18440-016 Applied Genetics p/n RHB
Clear E-Gel 18-Pak (1.2% agarose, no stain)	Invitrogen p/n G5518-01
SYBR Gold Nucleic Acid Gel Stain	Invitrogen p/n S11494
For possible use as a reference sample: Human Genomic DNA *or* Mouse Genomic DNA *or* Rat Genomic DNA	 Promega p/n G1521 (female) or p/n G1471 (male) Jackson Labs p/n 000664 (female and male) Harlan Sprague Dawley (custom)
Qiagen RNase A (100 mg/mL)	Qiagen p/n 19101
Qiagen Proteinase K (>600 mAU/mL, solution)	Qiagen p/n 19131
Qiagen DNeasy Blood & Tissue Kit	Qiagen p/n 69504
Sodium thiocyanate (NaSCN)	Sigma 467871-50G
Acetonitrile*	Sigma p/n 271004-1L
Ethanol (95% to 100% molecular biology grade)	Sigma p/n E7023-6x500ML
Tween 20	Sigma p/n P9416-50ML

* Optional components recommended if wash procedure B is selected.

Required Hardware and Software

- Pentium III 1.5 GHz or higher (Pentium 4 processor, 2.0 GHz or higher recommended)
- 2 GB RAM
- 20 GB available disk space (if saving images and result files locally)
- Windows 2000 SP2 or later, Windows XP SP2
- PC must be able to load MSDE 2000 release A Version 8.0
- Agilent Feature Extraction software v9.5
- Internet Explorer 5.5 or later
- Adobe Acrobat Reader 4.0 or later
- Virtual Memory - set at 2 GB

To access Virtual Memory settings in Windows XP:

1. Open the **Control Panel**.
2. Double-click **System**, and click the **Advanced** tab.
3. Click **Settings** (Performance) > **Advanced** > **Change** (Virtual Memory).
4. In the **Drive** list, click the drive that contains the paging file you want to change.
5. Under **Paging file size for selected drive**, type `2000` into the **Initial Size (MB)** box and click **Set** and then **OK**.

To access Virtual Memory settings in Windows 2000:

1. Open the **Control Panel**.
2. Double-click **System**, and click the **Advanced** tab.
3. Click **Performance Options** > **Change** (Virtual Memory).
4. In the **Drive** list, click the drive that contains the paging file you want to change.
5. Under **Paging file size for selected drive**, type `2000` into the **Initial Size (MB)** box and click **Set** and then **OK**.

2 DNA Isolation

Step 1. Paraffin Removal 19
Step 2. Proteinase K Treatment 20
Step 3. gDNA Extraction 21
Step 4. gDNA Quantitation and Quality Analysis 23
Supplemental Procedure - To isolate gDNA from frozen tissues 25

NOTE Agilent cannot guarantee microarray performance and does not provide technical support to those who use non-Agilent protocols in processing Agilent microarrays.

This chapter describes Agilent's recommended procedure to isolate genomic DNA (gDNA) from formalin-fixed paraffin-embedded (FFPE) samples and is based on the method described by van Beers et al. (Br J Cancer. 2006 Jan 30; 94(2):333-7). Determine the number of FFPE sections needed for your experiment based on the estimates summarized in Table 1 on page 18. One 20 micron FFPE section containing 1 cm^2 of tissue is estimated to generate a minimal yield of 500 ng of gDNA.

Agilent's array-based Comparative Genomic Hybridization (aCGH) application uses a "two-color" process to measure DNA copy number changes in an experimental sample relative to a reference sample. The type of sample used as a reference is a matter of experimental choice; however, many experimenters use normal commercial gDNA as a reference sample.

Table 1 Estimated number of 20 micron FFPE sections needed per microarray

Microarray format	gDNA input amount requirement [ng]	Estimated number of 20 micron FFPE sections
1x244K microarray	2000	4 to 5
2x105K microarray	1000	3
4x44K microarray	500	2
8x15K microarray	250	1

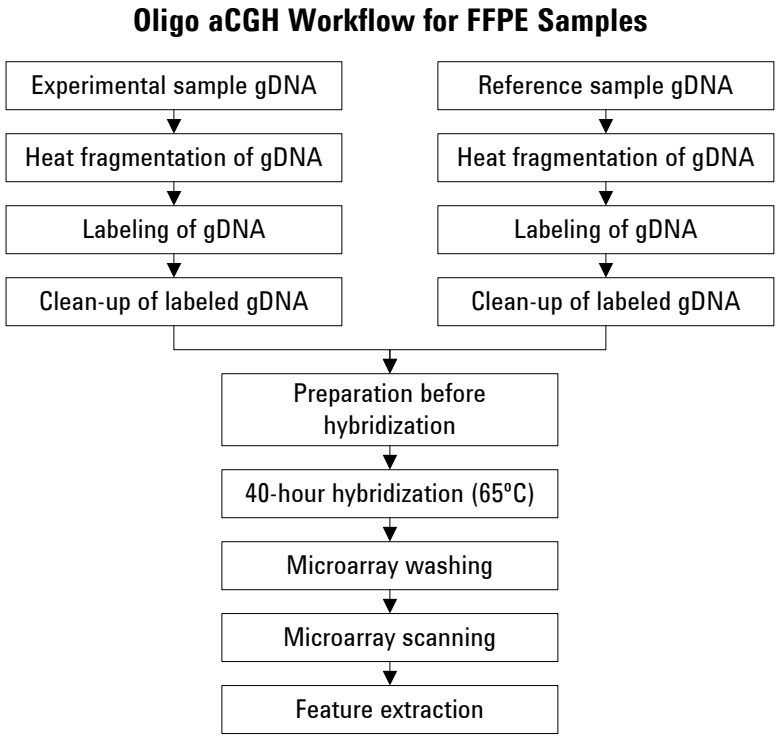


Figure 1 Workflow diagram for sample preparation and microarray processing.

Step 1. Paraffin Removal

1. Equilibrate a heat block or water bath to 90°C and a thermomixer to 37°C.
2. Place up to 5 20-micron FFPE sections into a 1.5 mL nuclease-free microfuge tube.
3. Prepare 10% Tween 20, by adding 100 µL Tween 20 to 900 µL of nuclease-free water. Solution can be prepared in advance and stored up to 6 months at room temperature.
4. Add 480 µL PBS and 20 µL 10% Tween 20 to the FFPE sections in the 1.5 mL nuclease-free microfuge tube.
5. Transfer the sample tube to a circulating water bath or heat block at 90°C. Incubate at 90°C for 10 minutes.
6. Spin immediately for 15 minutes at 10,000 x g in a microcentrifuge.
7. Place the sample tube on ice for 2 minutes.
8. Remove wax disc with a pipette tip or tweezers. Remove and discard the supernatant without disturbing the pellet.
9. Add 1 mL of 100% ethanol to the pellet and vortex briefly.
10. Spin for 5 minutes at 10,000 x g in a microcentrifuge.
11. Remove ethanol without disturbing the pellet and let the sample tube sit at room temperature with the lid open until residual ethanol has completely evaporated.
12. Prepare a 1M NaSCN solution by adding 10 g of NaSCN to 123 mL of nuclease free water. Solution can be prepared in advance and stored up to 1 month at room temperature.
13. Add 400 µL 1M NaSCN to the dry pellet and briefly mix on a vortex mixer.
14. Transfer the sample tube to a thermomixer at 37°C. Incubate overnight at 37°C shaking at 450 rpm.

Step 2. Proteinase K Treatment

1. Equilibrate a thermomixer to 55°C.
2. Transfer the sample tube to a microcentrifuge. Spin for 20 minutes at 10,000 x g.
3. Remove and discard the supernatant without disturbing the pellet.
4. Add 400 µL PBS to the pellet and vortex briefly.
5. Spin again for 20 minutes at 10,000 x g in a microcentrifuge.
6. Remove and discard the supernatant without disturbing the pellet.
7. Add 360 µL of Qiagen buffer ATL (supplied with Qiagen DNeasy Blood & Tissue Kit).
8. Add 40 µL proteinase K (supplied with Qiagen DNeasy Blood & Tissue Kit), mix well on a vortex mixer, and incubate overnight in a thermomixer at 55°C shaking at 450 rpm.
9. Transfer the sample tube to a microcentrifuge. Spin for 30 seconds at 6,000 x g to drive the contents off the walls and lid.
10. Add 40 µL proteinase K, mix well on a vortex mixer, and incubate in a thermomixer for approximately 6 to 8 hours at 55°C shaking at 450 rpm.
11. At the end of the day, transfer the sample tube to a microcentrifuge and spin for 30 seconds at 6,000 x g to drive the contents off the walls and lid.
12. Add 40 µL proteinase K, mix well on a vortex mixer, and incubate overnight in a thermomixer at 55°C shaking at 450 rpm.

Step 3. gDNA Extraction

1. Equilibrate a heat block or water bath to 70°C.
2. Let samples cool to room temperature and spin in a microcentrifuge for 30 seconds at 6,000 x g to drive the contents off the walls and lid.
3. Add 8 µL of RNase A (100 mg/mL), mix on a vortex mixer, and incubate for 2 minutes at room temperature. Transfer the sample tube to a microcentrifuge and spin for 30 seconds at 6,000 x g to drive the contents off the walls and lid.
4. Add 400 µL Buffer AL (supplied with Qiagen DNeasy Blood & Tissue Kit), mix thoroughly on a vortex mixer, and incubate in a circulating water bath or heat block at 70°C for 10 minutes. Transfer the sample tube to a microcentrifuge and spin for 30 seconds at 6,000 x g to drive the contents off the walls and lid.
5. Add 440 µL 100% ethanol, and mix thoroughly on a vortex mixer. Transfer the sample tube to a microcentrifuge and spin for 30 seconds at 6,000 x g to drive the contents off the walls and lid.
6. Place two DNeasy Mini spin columns in two clean 2 mL collection tubes (provided). Split the entire sample mixture onto two DNeasy Mini spin columns (i.e. 660 µL each).

NOTE Use 2 DNeasy Mini spin columns per sample to prevent clogging.

7. Spin in a microcentrifuge for 1 minute at 6,000 x g. Discard the flow-through and collection tube. Place the DNeasy Mini spin columns in fresh 2 mL collection tubes (provided).
8. Before using for the first time, prepare Buffer AW1 by adding 100% ethanol to the Buffer AW1 bottle (supplied with Qiagen DNeasy Blood & Tissue Kit; see bottle label for volume). Mark appropriate check box to indicate that ethanol was added to the bottle.
9. Add 500 µL Buffer AW1 onto each spin column, and centrifuge for 1 minute at 6,000 x g. Discard the flow-through and collection tube. Place the DNeasy Mini spin columns in fresh 2 mL collection tubes (provided).

10 Prepare a fresh 80% ethanol solution by adding 40 mL 100% ethanol to 10 mL nuclease-free water.

CAUTION Do *not* use Buffer AW2 supplied with the Qiagen DNeasy Blood & Tissue Kit for the subsequent step because salt from Buffer AW2 will interfere with the subsequent labeling reaction.

11 Add 500 µL 80% ethanol onto each column, and spin in a microcentrifuge for 3 minutes at 20,000 x g to dry the column membrane. Discard the flow-through and collection tube.

12 Place the DNeasy Mini spin column in a clean 1.5 mL microcentrifuge tube, and add 50 µL of nuclease free water directly to the center of each spin column.

13 Let stand at room temperature for 1 minute, and then spin in a microcentrifuge for 1 minute at 6,000 x g to elute the DNA.

14 Combine the purified DNA from the same sample in one microcentrifuge tube with a final total volume of 100 µL.

Step 4. gDNA Quantitation and Quality Analysis

Accurate assessment of gDNA quantity and quality are crucial to the success of an Agilent Oligo aCGH experiment. High quality gDNA should be free of contaminants such as carbohydrates, proteins, and traces of organic solvents, and should also be intact with minimal degradation. gDNA isolated from FFPE samples typically exhibits varying degrees of degradation depending on the age of the tissue and the paraffin embedding protocol used.

Use the NanoDrop ND-1000 UV-VIS Spectrophotometer (or equivalent) to assess gDNA concentration and purity. Use the agarose gel electrophoresis to assess gDNA intactness and the average molecular weight for each sample. This information will be important for the subsequent labeling reaction.

1. Select **Nucleic Acid Measurement**, then select **Sample Type** to be **DNA-50**.
2. Use 1.5 µL of nuclease free water to blank the instrument.
3. Use 1.5 µL of each gDNA sample to measure DNA concentration. Record the gDNA concentration (ng/µL) for each sample. Calculate the yield (µg) by multiplying DNA concentration (ng/µL) by the sample volume (that is, 100 µL) and dividing by 1000.
4. Record the $\mathbf{A_{260}/A_{280}}$ and $\mathbf{A_{260}/A_{230}}$ ratios. High-quality gDNA samples should have an $\mathbf{A_{260}/A_{280}}$ ratio of 1.8 to 2.0, indicating the absence of contaminating proteins, and an $\mathbf{A_{260}/A_{230}}$ ratio of >2.0, indicating the absence of other organic compounds such as guanidinium isothiocyanate, alcohol and phenol as well as cellular contaminants such as carbohydrates.
5. Load 20 ng gDNA for each sample in a volume of 10 µL nuclease-free water in the well of a single-comb 1.2% Clear E-Gel. (No need to add loading buffer in this system).
6. As a control, load 20 ng of commercial Human Genomic DNA in a volume of 10 µL nuclease free water in one of the wells of the E-Gel.
7. Mix 5 µL TrackIt 1 Kb DNA Ladder with 95 µL deionized water and load 10 µL of the diluted ladder in one of the wells of the E-Gel.
8. Run the gel for 30 minutes as described in Invitrogen's instructions.
9. Open the gel cassette with E-Gel Opener as described in Invitrogen’s instructions.

10 Stain the gel with SYBR Gold Nucleic Acid Gel Stain (diluted 1:10,000 by adding 10 µL of SYBR Gold Nucleic Acid Gel Stain to 100 mL of nuclease-free water) in a plastic tray for 15 minutes.

11 Visualize the gel on the UV-transilluminator using a SYBR Gold photographic filter.

Supplemental Procedure - To isolate gDNA from frozen tissues

This supplemental step describes Agilent's recommended procedure to isolate genomic DNA (gDNA) from frozen tissue using the Qiagen DNeasy Blood & Tissue Kit (p/n 69504).

1. Equilibrate a thermomixer to 55°C and heat block or water bath to 70°C.
2. Cut up to 25 mg frozen tissue (up to 10 mg for spleen tissue) into small pieces, place into a 1.5 mL microfuge tube, and add 180 µL Buffer ATL.
3. Add 20 µL proteinase K, mix well on a vortex mixer, and incubate in a thermomixer at 55°C shaking at 450 rpm until the tissue is completely lysed.

   Lysis time varies depending on the type of tissue processed. Usually lysis is complete in 1 to 3 hours. If it is more convenient, samples can be lysed overnight.
4. Let the sample cool to room temperature and spin in a microcentrifuge for 30 seconds at 6,000 x g to drive the contents off the walls and lid.
5. Add 4 µL of RNase A (100 mg/mL), mix on a vortex mixer, and incubate for 2 minutes at room temperature. Spin in a microcentrifuge for 30 seconds at 6,000 x g to drive the contents off the walls and lid.
6. Add 200 µL Buffer AL to each sample, mix thoroughly on a vortex mixer, and incubate at 70°C for 10 minutes in a heat block or water bath. Spin in a microcentrifuge for 30 seconds at 6,000 x g to drive the contents off the walls and lid.
7. Add 200 µL 100% ethanol to each sample, and mix thoroughly on a vortex mixer. Spin in a microcentrifuge for 30 seconds at 6,000 x g to drive the contents off the walls and lid.
8. Transfer the sample mixture onto a DNeasy Mini spin column placed in a 2 mL collection tube (provided). Centrifuge at 6,000 x g for 1 minute. Discard the flow-through and collection tube. Place the DNeasy Mini spin column in a new 2 mL collection tube (provided).
9. Before using for the first time, prepare Buffer AW1 by adding 100% ethanol to the Buffer AW1 bottle (supplied with Qiagen DNeasy Blood & Tissue Kit; see bottle label for volume). Mark the appropriate check box to indicate that ethanol was added to the bottle.

10 Add 500 µL Buffer AW1 onto the column, and spin in a microcentrifuge for 1 minute at 6,000 x g. Discard the flow-through and collection tube. Place the DNeasy Mini spin column in a new 2 mL collection tube (provided).

11 Prepare a fresh 80% ethanol solution by adding 40 mL 100% ethanol to 10 mL nuclease-free water.

CAUTION Do not use Buffer AW2 supplied with the Qiagen DNeasy Blood & Tissue Kit for the subsequent step because salt from Buffer AW2 will interfere with the subsequent labeling reaction.

12 Add 500 µL 80% ethanol onto the column, and centrifuge for 3 minutes at 20,000 x g to dry the DNeasy membrane. Discard the flow-through and collection tube.

13 Place the DNeasy Mini spin column in a clean 1.5 mL microcentrifuge tube, and pipet 200 µL of nuclease free water directly onto the center of the DNeasy column membrane.

14 Incubate at room temperature for 1 minute, and then spin in a microcentrifuge for 1 minute at 6,000 x g to elute the DNA.

15 Repeat elution with nuclease free water once as described in step 13 and step 14. Combine the duplicate samples in one microcentrifuge tube for a final volume of 400 µL.

16 Measure gDNA concentration and purity, and analyze on an agarose gel as described in "Step 4. gDNA Quantitation and Quality Analysis" on page 23.

3 DNA Labeling

Step 1. Preparation of gDNA Before Labeling 28
Step 2. Heat Fragmentation 29
Step 3. ULS Labeling 29
Step 4. Free Dye Removal 32

The Agilent Oligo aCGH Labeling Kit for FFPE Samples offers a one-step non-enzymatic procedure to differentially label gDNA samples with fluorescent dyes. For Agilent's Oligo aCGH application, the experimental sample is labeled with one dye while the reference sample is labeled with the other dye. The “polarity” of the sample labeling is a matter of experimental choice.

Equal amounts of gDNA for both the experimental and reference channels should be used. The required gDNA input amount depends on the microarray format used (see Table 2 on page 28).

CAUTION

gDNA samples need to be clean of salt and other (wash) buffer components as well as divalent cations (e.g. Mg 2+) which can disturb the subsequent labeling efficiency. Follow the DNA isolation procedure described in Chapter 2, “DNA Isolation”. Failure to do so will result in unsatisfactory microarray results.

Table 2 Requirement of gDNA Input Amount and Volume per Microarray

Microarray format	gDNA input amount [ng]	Volume of gDNA [µL]	Minimum gDNA concentration [ng/µL]
1x244K microarray	2000	16	125
2x105K microarray	1000	17	59
4x44K microarray	500	8	62.5
8x15K microarray	250	8	32

Step 1. Preparation of gDNA Before Labeling

1 If the gDNA concentration is less than 125 ng/µL (for 1x244K microarrays), 59 ng/µL (for 2x105K microarrays), 62.5 ng/µL (for 4x44K microarrays), or 32 ng/µL (for 8x15K microarrays), concentrate the sample using a speed-vac before you proceed to the heat fragmentation.

2 Add 2 µg of gDNA and the appropriate volume of nuclease-free water to bring to a final volume of 16 µL (for 1x244K microarrays), 1 µg of gDNA and the appropriate volume of nuclease-free water to bring to a final volume of 17 µL (for 2x105K microarrays), 500 ng of gDNA and the appropriate volume of nuclease-free water to bring to a final volume of 8 µL (for 4x44K microarrays), or 250 ng of gDNA and the appropriate volume of nuclease-free water to bring to a final volume of 8 µL (for 8x15K microarrays) in a 0.2 mL nuclease-free PCR tube.

Step 2. Heat Fragmentation

1 Estimate the average molecular weight for each gDNA sample based on the agarose gel analysis (see "DNA Isolation" on page 17).

2 Fragment the gDNA samples by incubating at 95°C in a PCR machine with heated lid for the time period indicated in Table 3.

3 Transfer the sample tubes to ice and incubate on ice for 3 minutes.

4 Spin in a microcentrifuge for 30 seconds at 6,000 × g to drive the contents off the walls and lid.

Table 3 Length of heat fragmentation

Average molecular weight	Sample type	Fragmentation time
> 10 KB	Reference sample with intact gDNA	10 minutes
> 7 KB	Some fresh FFPE samples	5 minutes
< 7 KB	Most FFPE samples	No fragmentation

Step 3. ULS Labeling

NOTE ULS-Cy3 and ULS-Cy5 are light sensitive. Minimize light exposure throughout the labeling procedure.

1 Prepare the Labeling Master Mix by mixing the components in Table 4, Table 5, Table 6, or Table 7 on ice based on the microarray format of choice. Avoid pipetting volumes less than 2 µL to ensure accuracy.

Table 4 Preparation of Labeling Master Mix (for 1x244K microarray)

Components	Per reaction (µL)	Per slide (µL)
ULS-Cy3 or ULS-Cy5	2	2
10 x labeling solution	2	2
Final volume of Labeling Master Mix	**4**	**4**

Table 5 Preparation of Labeling Master Mix (for 2x105K microarray)

Components	Per reaction (µL)	Per slide (µL) (including excess)
ULS-Cy3 or ULS-Cy5	1	2.5
10 x labeling solution	2	5
Final volume of Labeling Master Mix	**3**	**7.5**

Table 6 Preparation of Labeling Master Mix (for 4x44K microarray)

Components	Per reaction (µL)	Per slide (µL) (including excess)
Nuclease-free water	0.5	2.5
ULS-Cy3 or ULS-Cy5	0.5	2.5
10 x labeling solution	1	5
Final volume of Labeling Master Mix	**2**	**10**

Table 7 Preparation of Labeling Master Mix (for 8x15K microarray)

Components	Per reaction (µL)	Per slide (µL) (including excess)
Nuclease-free water	0.75	7.5
ULS-Cy3 or ULS-Cy5	0.25	2.5
10 x labeling solution	1	10
Final volume of Labeling Master Mix	**2**	**20**

2 Add the appropriate amount of Labeling Master Mix to each PCR tube containing the gDNA to make a total volume as listed in Table 8. Mix well by gently pipetting up and down.

Table 8 Amount of Labeling Master Mix to add

Microarray format	Volume of Labeling Master Mix	Volume of gDNA	Total volume
1x244K microarray	4 µL	16 µL	20 µL
2x105K microarray	3 µL	17 µL	20 µL
4x44K microarray	2 µL	8 µL	10 µL
8x15K microarray	2 µL	8 µL	10 µL

3 Transfer PCR tubes to a PCR machine with heated lid and incubate at 85°C for 30 minutes.

4 Transfer the samples to ice and incubate on ice for 3 minutes.

5 Spin in a microcentrifuge for 1 minute at 6,000 × g to drive the contents off the walls and lid.

Labeled gDNA can be stored on ice until dye removal using the Agilent KREApure columns.

6 **For 4x44K microarrays samples only**: add 10 µL of nuclease free water to each PCR tube to make a total volume of 20 µL.

CAUTION Do not add nuclease free water to the 8x15K microarray sample tubes as dilution of the samples will prevent accurate measurement of gDNA concentration and Degree of Labeling by Nanodrop.

Step 4. Free Dye Removal

Free ULS-Cy3 or ULS-Cy5 can interfere with the subsequent microarray experiment and increase background noise if they are not efficiently removed prior to hybridization. Agilent KREApure columns are provided to effectively remove free ULS dye.

NOTE Use the same microcentrifuge and centrifuge speed and length for all three spinning steps (step 4, step 7 and step 11).

1. Resuspend Agilent KREApure column material by briefly mixing on a vortex mixer.
2. Loosen cap ¼ turn and snap off the bottom closure.
3. Place the Agilent KREApure column in a 2 mL collection tube.
4. Pre-spin the Agilent KREApure column in a microcentrifuge for 1 minute at maximum speed (minimum 16,000 x g).
5. Discard the flow-through and place the Agilent KREApure column back to the same collection tube.
6. Add 300 µL nuclease free water to the Agilent KREApure column.
7. Spin again in a microcentrifuge for 1 minute at maximum speed (minimum 16,000 x g).
8. Discard the flow-through and collection tube.
9. Transfer Agilent KREApure column to a clean 1.5 mL heat-resistant microcentrifuge tube.
10. Add ULS-labeled gDNA (20 µL or 10 µL for 8x15K microarray samples) onto Agilent KREApure column.
11. Spin in a microcentrifuge for 1 minute at maximum speed (minimum 16,000 x g) to collect the purified labeled gDNA in the collection tube.
12. Use the NanoDrop ND-1000 UV-VIS Spectrophotometer to measure the Degree of Labeling:
    a. Select **MicroArray Measurement**, then select **Sample Type** to be **DNA-50**.
    b. Blank the instrument with 1.5 µL of 1x labeling solution.
    c. Use 1.5 µL of each labeled gDNA sample for quantitation. Measure the absorbance at A_{260} nm (DNA), A_{550} nm (Cy3), and A_{650} nm (Cy5).

d Calculate the Degree of Labeling of the labeled gDNA:

$$\text{Degree of Labeling} = \frac{340 \times \text{pmol per } \mu\text{L dye}}{\text{ng per } \mu\text{L genomic DNA} \times 1000} \times 100\%$$

As a general guideline, an optimal Degree of Labeling lies between 1.5% and 2.5%.

13 Combine the appropriate ULS-Cy5-labeled sample and ULS-Cy3-labeled sample for a total volume of 37 µL (for 1x244K, 2x105K and 4x44K microarrays) or 17 µL (for 8x15K microarrays) in a 1.5-mL heat-resistant microfuge tube.

Labeled gDNA can be stored overnight at -20°C in the dark.

4 Microarray Processing and Feature Extraction

Step 1. Preparation of Labeled Genomic DNA for Hybridization 36
Step 2. Microarray Hybridization 41
Step 3. Wash Preparation 43
Step 4. Microarray Washing 46
Step 5. Microarray Scanning using Agilent or GenePix Scanner 51
Step 6. Data Extraction using Feature Extraction Software 53

Microarray processing consists of hybridization, washing, and scanning.

Feature Extraction is the process by which data is extracted from the scanned microarray image (.tif), allowing researchers to measure DNA copy number changes in their experiments in conjunction with Agilent CGH Analytics Software.

Step 1. Preparation of Labeled Genomic DNA for Hybridization

1 Prepare the **10X CGH Blocking Agent**:

   a Add 1350 µL of nuclease-free water to the vial containing lyophilized 10X CGH Blocking Agent (supplied with Agilent Oligo aCGH Hybridization Kit).

   b Mix briefly on a vortex mixer and leave at room temperature for 60 minutes to reconstitute sample before use or storage.

   c

2 Equilibrate water baths or heat blocks to 95°C and 37°C.

3 Use a speed-vac to concentrate the combined Cy5- and Cy3-labeled gDNA mixture to the final volume listed below according to the microarray format:

1x244K microarray	28 µL
2x105K microarray	14 µL
4x44K microarray	12 µL
8x15K microarray	5 µL

4 Prepare the Hybridization Master Mix by mixing the components in the table below according to the microarray format:

Table 9 Preparation of Hybridization Master Mix for 1x244K microarray

Component	Volume per hybridization	Volume per slide
Cot-1 DNA (1.0 mg/mL)*	50 µL	50 µL
Agilent 10X CGH Blocking Agent†	52 µL	52 µL
Agilent 2X CGH Hybridization Buffer†	260 µL	260 µL
Final volume of Hybridization Master Mix	**362 µL**	**362 µL**

* Use Cot-1 DNA from the appropriate species.

† Supplied with Agilent Oligo aCGH Hybridization Kit

Table 10 Preparation of Hybridization Master Mix for 2x105K microarray

Component	Volume per hybridization	Volume per slide (including excess)
Cot-1 DNA (1.0 mg/mL)*	25 µL	62.5 µL
Agilent 10X CGH Blocking Agent†	26 µL	65 µL
Agilent 2X CGH Hybridization Buffer†	130 µL	325 µL
Final volume of Hybridization Master Mix	**181 µL**	**452.5 µL**

* Use Cot-1 DNA from the appropriate species.

† Supplied with Agilent Oligo aCGH Hybridization Kit

Table 11 Preparation of Hybridization Master Mix for 4x44K microarray

Component	Volume per hybridization	Volume per slide (including excess)
Cot-1 DNA (1.0 mg/mL)*	5 µL	25 µL
Agilent 10X CGH Blocking Agent†	11 µL	55 µL
Agilent 2X CGH Hybridization Buffer†	55 µL	275 µL
Final volume of Hybridization Master Mix	**71 µL**	**355 µL**

* Use Cot-1 DNA from the appropriate species.

† Supplied with Agilent Oligo aCGH Hybridization Kit

Table 12 Preparation of Hybridization Master Mix for 8x15K microarray

Component	Volume per hybridization	Volume per slide (including excess)
Cot-1 DNA (1.0 mg/mL)*	2 µL	20 µL
Agilent 10X CGH Blocking Agent†	4.5 µL	45 µL
Agilent 2X CGH Hybridization Buffer†	22.5 µL	225 µL
Final volume of Hybridization Master Mix	**29 µL**	**290 µL**

* Use Cot-1 DNA from the appropriate species.

† Supplied with Agilent Oligo aCGH Hybridization Kit

5 Add the appropriate volume of the Hybridization Master Mix to each 1.5 mL microfuge tube containing the labeled gDNA to make the total volume listed in Table 13.

Table 13 Volume of Hybridization Master Mix per hybridization

Microarray format	Volume of Hybridization Master Mix	Total volume
1x244K microarray	362 µL	390 µL
2x105K microarray	181 µL	195 µL
4x44K microarray	71 µL	83 µL
8x15K microarray	29 µL	34 µL

6 Mix the sample by pipetting up and down, and then quickly spin in a microcentrifuge to drive the contents off the walls and lid.

7 Transfer sample tubes to a circulating water bath or heat block at 95°C. Incubate at 95°C for 3 minutes.

8 Immediately transfer sample tubes to a circulating water bath or heat block at 37°C. Incubate at 37°C for 30 minutes.

9 Remove sample tubes from the water bath or heat block. Quickly spin in a microcentrifuge to drive the contents off the walls and lid.

10 Add the appropriate volume of Agilent-CGHblock (supplied with the Agilent Oligo aCGH Labeling Kit for FFPE Samples) to each 1.5 mL microfuge tube containing the labeled gDNA and Hybridization Master Mix to make the final volume of hybridization sample mixture listed in Table 14.

Mix well by pipetting up and down.

Table 14 Volume of Agilent-CGHblock per hybridization

Microarray format	Volume of Agilent-CGHblock	Final volume of hybridization sample mixture
1x244K microarray	130 µL	520 µL
2x105K microarray	65 µL	260 µL
4x44K microarray	27 µL	110 µL
8x15K microarray	11 µL	45 µL

11 Spin 1 minute at 17,900 × g in a microcentrifuge to collect the sample at the bottom of the reaction tube.

Step 2. Microarray Hybridization

NOTE

Familiarize yourself with the assembly and disassembly instructions for use with the Agilent Microarray Hybridization Chamber and gasket slides. Please refer to the Agilent Microarray Hybridization Chamber User Guide (G2534-90001) for in-depth instructions on how to load samples, assemble and disassemble chambers, as well as other helpful tips. This user guide is available with the purchase of Agilent Microarray Hybridization Chamber Kit (G2534A) and can also be downloaded from the Agilent Web site at www.agilent.com/chem/dnamanuals-protocols. Practice slide kits are also available.

Microarray Handling Tips

Each microarray is printed on the side of the glass slide containing the "Agilent"-labeled barcode. This side is called the "active side". The numeric barcode is on the "inactive side" of the glass slide.

The hybridization sample mixture is applied directly to the gasket slide and not to the active side of the microarray slide. Then the active side of the microarray slide is placed on top of the gasket slide to form a "sandwich slide pair".

To avoid damaging the microarray, always handle glass slides carefully by their edges. Wear powder-free gloves. Never touch the surfaces of the slides. If you do, you may cause irreparable damage to the microarray.

Never allow the microarray surface to dry out during the hybridization process and washing steps.

Hybridization Assembly

1. Load a clean gasket slide into the Agilent SureHyb chamber base with the gasket label facing up and aligned with the rectangular section of the chamber base. Ensure that the gasket slide is flush with the chamber base and is not ajar.
2. Slowly dispense 490 µL (for 1x244K microarray), 245 µL (for 2x105K microarray), 100 µL (for 4x44K microarray) or 40 µL (for 8x15K microarray) of hybridization sample mixture onto the gasket well in a "drag and dispense" manner. For multi-pack microarray formats (i.e. 2x105K, 4x44K or 8x15K microarray), load all gasket wells before placing the microarray slide.

3 Place a microarray slide “active side” down onto the gasket slide, so the numeric barcode side is facing up and the “Agilent”-labeled barcode is facing down. Assess that the sandwich-pair is properly aligned.

4 Place the SureHyb chamber cover onto the sandwiched slides and slide the clamp assembly onto both pieces.

5 Hand-tighten the clamp onto the chamber.

6 Vertically rotate the assembled chamber to wet the slides and assess the mobility of the bubbles. Tap the assembly on a hard surface if necessary to move stationary bubbles.

7 Place assembled slide chamber in the rotator rack in a hybridization oven set to 65°C. Set your hybridization rotator to rotate at 20 rpm.

8 Hybridize at 65°C for 40 hours.

CAUTION

If you are not loading all the available positions on the hybridization rotator rack, be sure to *balance* the loaded hybridization chambers on the rack similar to a centrifuge to prevent unnecessary strain on the oven motor.

For more information on the effects of hybridization temperature and time, as well as the rotation speed on the final microarray results, please refer to the application note titled “60-mer Oligo-Based Comparative Genomic Hybridization” (publication 5989-4848EN) from the Agilent Web site at www.agilent.com/chem/dnaapplications.

Step 3. Wash Preparation

NOTE Cyanine 5 has been shown to be sensitive to ozone degradation. Ozone levels as low as 5 ppb (approximately 10 µg/m^3) can affect Cyanine 5 signal and compromise microarray results. The Agilent Stabilization and Drying Solution is designed to protect against ozone-induced degradation of Cyanine dyes. Use this solution when working with Agilent oligo-based microarrays in high ozone environments.

Before you begin, determine which wash procedure to use:

- Use "Wash Procedure A (without Stabilization and Drying Solution)" on page 46 if you are washing slides in an ozone-controlled environment, and the ozone level is 5 ppb or less.
- Use "Wash Procedure B (with Stabilization and Drying Solution)" on page 48 if you are washing slides in an environment in which the ozone level exceeds 5 ppb.

Equipment Preparation

CAUTION Do not use detergent to wash the staining dishes as some detergents may leave fluorescent residue on the dishes. If you do, you must ensure that all traces are removed by thoroughly rinsing with Milli-Q water.

- Always use clean equipment when conducting the wash procedures.
- Use only dishes that are designated and dedicated for use in Agilent oligo aCGH experiments.

- Cleaning with Milli-Q Water Wash

  Rinse slide-staining dishes, slide racks and stir bars thoroughly with high-quality Milli-Q water before use and in between washing groups.

  a Run copious amounts of Milli-Q water through the slide-staining dishes, slide racks and stir bars.

  b Empty out the water collected in the dishes at least five times.

  c Repeat step a and step b until all traces of contaminating material are removed.

- Cleaning with Acetonitrile Wash (Wash Procedure B Only)

Acetonitrile wash removes any remaining residue of Agilent Stabilization and Drying Solution from slide-staining dishes, slide racks and stir bars that were used in previous experiments with "Wash Procedure B (with Stabilization and Drying Solution)" on page 48.

WARNING **Do acetonitrile washes in a vented fume hood. Acetonitrile is highly flammable and toxic.**

---

a Add the slide rack and stir bar to the slide-staining dish, and transfer to a magnetic stir plate.

b Fill the slide-staining dish with 100% acetonitrile.

c Turn on the magnetic stir plate and adjust the speed to a setting of 4 (medium speed).

d Wash for 5 minutes at room temperature.

e Discard the acetonitrile as is appropriate for your site.

f Repeat step a through step e.

g Air dry everything in the vented fume hood.

h Continue with the Milli-Q water wash as previously instructed.

Prewarming Oligo aCGH Wash Buffer 2 (Overnight)

The temperature of Oligo aCGH Wash Buffer 2 must be at 37°C for optimal performance.

1 Add the volume of buffer required to a disposable plastic bottle and warm overnight in an incubator or circulating water bath set to 37°C.

2 Put a slide-staining dish into a 1.5 L glass dish three-fourths filled with water and warm to 37°C by storing overnight in an incubator set to 37°C.

Prewarming Stabilization and Drying Solution (Wash Procedure B Only)

The Agilent Stabilization and Drying Solution contains an ozone scavenging compound dissolved in acetonitrile. The compound in solution is present in saturating amounts and may precipitate from the solution under normal storage conditions. If the solution shows visible precipitation, warming of the solution will be necessary to redissolve the compound. Washing slides using Stabilization and Drying Solution showing visible precipitation will have profound adverse affects on array performance.

WARNING **The Agilent Stabilization and Drying Solution is a flammable liquid. Warming the solution will increase the generation of ignitable vapors. Use gloves and eye/face protection in every step of the warming procedures.**

WARNING **Do not use an open flame or a microwave. Do not increase temperature rapidly. Warm and mix the material away from ignition sources.**

WARNING **Failure to follow the outlined process will increase the potential for fire, explosion, and possible personal injury.**

1 Warm the solution slowly in a water bath set to 37°C to 40°C in a closed container with sufficient head space to allow for expansion. Warm the solution only in a controlled and contained area that meets local fire code requirements.

   The original container can be used to warm the solution. Container volume is 700 mL and contains 500 mL of liquid. If a different closed container is used, maintain or exceed this headspace/liquid ratio. The time needed to completely redissolve the precipitate is dependent on the amount of precipitate present, and may require overnight warming if precipitation is heavy.

2 Gently shake the container to obtain a homogenous solution.

3 After the precipitate is completely dissolved, allow the solution to equilibrate to room temperature prior to use.

CAUTION Do not filter the Stabilization and Drying solution, or the concentration of the ozone scavenger may vary.

Step 4. Microarray Washing

- Use "Wash Procedure A (without Stabilization and Drying Solution)" on page 46 if you are washing microarray slides in an ozone-controlled environment, and the ozone level is 5 ppb or less.
- Use "Wash Procedure B (with Stabilization and Drying Solution)" on page 48 if you are washing microarray slides in an environment in which the ozone level exceeds 5 ppb.

Wash Procedure A (without Stabilization and Drying Solution)

Always use fresh Oligo aCGH Wash Buffer 1 and Oligo aCGH Wash Buffer 2 for each wash group (up to five slides).

Table 15 lists the wash conditions for the Wash Procedure A without Stabilization and Drying Solution.

Table 15 Wash conditions

	Dish	Wash buffer	Temperature	Time
Disassembly	#1	Oligo aCGH Wash Buffer 1	Room temperature	
1st wash	#2	Oligo aCGH Wash Buffer 1	Room temperature	5 minutes
2nd wash	#3	Oligo aCGH Wash Buffer 2	37°C	1 minute

1 Completely fill slide-staining dish #1 with Oligo aCGH Wash Buffer 1 at room temperature.

2 Place a slide rack into slide-staining dish #2. Add a magnetic stir bar. Fill slide-staining dish #2 with enough Oligo aCGH Wash Buffer 1 at room temperature to cover the slide rack. Place this dish on a magnetic stir plate.

3 Put the prewarmed 1.5 L glass dish filled with water and containing slide-staining dish #3 on a magnetic stir plate with heating element. Fill the slide-staining dish #3 approximately three-fourths full with Oligo aCGH Wash Buffer 2 (warmed to 37°C). Add a magnetic stir bar. Turn on the heating element and maintain temperature of Oligo aCGH Wash Buffer 2 at 37°C; monitor using a thermometer.

4 Remove one hybridization chamber from incubator and record time. Record whether bubbles formed during hybridization and if all bubbles are rotating freely.

5 Prepare the hybridization chamber disassembly.

   a Place the hybridization chamber assembly on a flat surface and loosen the thumbscrew, turning counter-clockwise.

   b Slide off the clamp assembly and remove the chamber cover.

   c With gloved fingers, remove the array-gasket sandwich from the chamber base by grabbing the slides from their ends. Keep the microarray slide numeric barcode facing up as you quickly transfer the sandwich to slide-staining dish #1.

   d Without letting go of the slides, submerge the array-gasket sandwich into slide-staining dish #1 containing Oligo aCGH Wash Buffer 1.

6 With the sandwich completely submerged in Oligo aCGH Wash Buffer 1, pry the sandwich open from the barcode end only. Do this by slipping one of the blunt ends of the forceps between the slides and then gently turn the forceps upwards or downwards to separate the slides. Let the gasket slide drop to the bottom of the staining dish. Remove the microarray slide and place into slide rack in the slide-staining dish #2 containing Oligo aCGH Wash Buffer 1 at room temperature. Minimize exposure of the slide to air. *Touch only the barcode portion of the microarray slide or its edges!*

7 Repeat step 4 through step 6 for up to four additional slides in the group. A maximum of five disassembly procedures yielding five microarray slides is advised at one time in order to facilitate uniform washing.

8 When all slides in the group are placed into the slide rack in slide-staining dish #2, stir using setting 4 for 5 minutes. Adjust the setting to get good but not vigorous mixing.

9 Transfer slide rack to slide-staining dish #3 containing Oligo aCGH Wash Buffer 2 at 37°C, and stir using setting 4 for 1 minute.

10 Slowly remove the slide rack trying to minimize droplets on the slides. It should take 5 to 10 seconds to remove the slide rack.

11 Discard used Oligo aCGH Wash Buffer 1 and Oligo aCGH Wash Buffer 2.

12 Repeat step 1 through step 11 for the next group of five slides using fresh Oligo aCGH Wash Buffer 1 and Oligo aCGH Wash Buffer 2 pre-warmed to 37°C.

13 Scan slides immediately to minimize impact of environmental oxidants on signal intensities. If necessary, store slides in the original slide boxes in a N_2 purge box, in the dark.

Wash Procedure B (with Stabilization and Drying Solution)

Cy5 is susceptible to degradation by ozone. Use this wash procedure if the ozone level exceeds 5 ppb in your laboratory.

Always use fresh Oligo aCGH Wash Buffer 1 and Oligo aCGH Wash Buffer 2 for each wash group (up to five slides).

The acetonitrile (dish #4) and Stabilization and Drying Solution (dish #5) below may be reused for washing up to 4 batches of 5 slides (total 20 slides).

WARNING **The Stabilization and Drying Solution must be set-up in a fume hood. Place the Wash Buffer 1 and Wash Buffer 2 set-up areas close to, or preferably in, the same fume hood. Use gloves and eye/face protection in every step of the washing procedure.**

Table 16 lists the wash conditions for the Wash Procedure B with Stabilization and Drying Solution.

Table 16 Wash conditions

	Dish	Wash Buffer	Temperature	Time
Disassembly	#1	Oligo aCGH Wash Buffer 1	Room temperature	
1st wash	#2	Oligo aCGH Wash Buffer 1	Room temperature	5 minutes
2nd wash	#3	Oligo aCGH Wash Buffer 2	37°C	1 minute
Acetonitrile wash	#4	Acetonitrile	Room temperature	1 minute
3rd wash	#5	Stabilization and Drying Solution	Room temperature	30 seconds

1 Completely fill slide-staining dish #1 with Oligo aCGH Wash Buffer 1 at room temperature.

2 Place a slide rack into slide-staining dish #2. Add a magnetic stir bar. Fill slide-staining dish #2 with enough Oligo aCGH Wash Buffer 1 at room temperature to cover the slide rack. Place this dish on a magnetic stir plate.

3 Put the prewarmed 1.5 L glass dish filled with water and containing slide-staining dish #3 on a magnetic stir plate with heating element. Fill the slide-staining dish #3 approximately three-fourths full with Oligo aCGH Wash Buffer 2 (warmed to 37°C). Add a magnetic stir bar. Turn on the heating element and maintain temperature of Oligo aCGH Wash Buffer 2 at 37°C; monitor using a thermometer.

4 In the fume hood, fill slide-staining dish #4 approximately three-fourths full with acetonitrile. Add a magnetic stir bar and place this dish on a magnetic stir plate.

5 In the fume hood, fill slide-staining dish #5 approximately three-fourths full with Stabilization and Drying Solution. Add a magnetic stir bar and place this dish on a magnetic stir plate.

6 Remove one hybridization chamber from incubator and record time. Record whether bubbles formed during hybridization, and if all bubbles are rotating freely.

7 Prepare the hybridization chamber disassembly.

   a Place the hybridization chamber assembly on a flat surface and loosen the thumbscrew, turning counter-clockwise.

   b Slide off the clamp assembly and remove the chamber cover.

   c With gloved fingers, remove the array-gasket sandwich from the chamber base by grabbing the slides from their ends. Keep the microarray slide numeric barcode facing up as you quickly transfer the sandwich to slide-staining dish #1.

   d Without letting go of the slides, submerge the array-gasket sandwich into slide-staining dish #1 containing Oligo aCGH Wash Buffer 1.

8 With the sandwich completely submerged in Oligo aCGH Wash Buffer 1, pry the sandwich open from the barcode end only. Do this by slipping one of the blunt ends of the forceps between the slides and then gently turn the forceps upwards or downwards to separate the slides. Let the gasket slide drop to the bottom of the staining dish. Remove the microarray slide and place into slide rack in the slide-staining dish #2 containing Oligo aCGH Wash Buffer 1 at room temperature. Minimize exposure of the slide to air. *Touch only the barcode portion of the microarray slide or its edges!*

9 Repeat step 6 through step 8 for up to four additional slides in the group. A maximum of five disassembly procedures yielding five microarray slides is advised at one time in order to facilitate uniform washing.

10 When all slides in the group are placed into the slide rack in slide-staining dish #2, stir using setting 4 for 5 minutes. Adjust the setting to get good but not vigorous mixing.

11 Transfer slide rack to slide-staining dish #3 containing Oligo aCGH Wash Buffer 2 at 37°C, and stir using setting 4 for 1 minute.

12 Remove the slide rack from Oligo aCGH Wash Buffer 2 and tilt the rack slightly to minimize wash buffer carry-over. Immediately transfer the slide rack to slide-staining dish #4 containing acetonitrile, and stir using setting 4 for 1 minute.

13 Transfer slide rack to slide-staining dish #5 filled with Stabilization and Drying Solution, and stir using setting 4 for 30 seconds.

14 Slowly remove the slide rack trying to minimize droplets on the slides. It should take 5 to 10 seconds to remove the slide rack.

NOTE

The acetonitrile and the Stabilization and Drying Solution may be reused for washing of up to four batches of five slides (that is, total 20 microarray slides). After each use, rinse the slide rack and the slide-staining dish that were in contact with the Stabilization and Drying Solution with acetonitrile followed by a rinse in Milli-Q water.

15 Repeat step 1 through step 14 for the next group of five slides using fresh Oligo aCGH Wash Buffer 1 and Oligo aCGH Wash Buffer 2 prewarmed to 37°C.

16 Scan slides immediately to minimize impact of environmental oxidants on signal intensities. If necessary, store slides in original slide boxes in a N_2 purge box, in the dark.

17 Dispose of acetonitrile and Stabilization and Drying Solution as flammable solvents.

Step 5. Microarray Scanning using Agilent or GenePix Scanner

Agilent Scanner Settings

Agilent Scanner Control software v7.0 is recommended for 5 µm scans of 1x244K, 2x105K, 4x44K and 8x15K density microarrays.

1 Assemble slides into appropriate slide holders:
   - For version B and A slide holders, place slide into slide holder with Agilent barcode facing up.
   - For version A slide holders, check that slides are seated parallel to the bottom of the slide holder.

2 Place assembled slide holders into scanner carousel.

3 Verify Default Scan Settings (click **Settings > Modify Default Settings**).
   - **Scan region** is set to **Scan Area (61 × 21.6 mm)**.
   - **Scan resolution (µm)** is set to **5** for 1x244K, 2x105K, 4x44K and 8x15K microarrays.
   - **Dye channel** is set to **Red & Green**.
   - **Green PMT** is set to **100%**.
   - **Red PMT** is set to **100%**.

4 Select settings for the automatic file naming.
   - **Prefix1** is set to **Instrument Serial Number**.
   - **Prefix2** is set to **Array Barcode**.

5 Clear the **eXtended Dynamic Range** check box.

6 Verify that the Scanner status in the main window says **Scanner Ready**.

7 Click **Scan Slot *m*-*n*** on the Scan Control main window where the letter m represents the Start slot where the first slide is located and the letter n represents the End slot where the last slide is located.

GenePix Scanner Settings

Only the GenePix 4000B scanner is supported for scanning Agilent CGH microarrays.

- Refer to the manufacturer's user guide for appropriate scanner settings.
- Refer to "Agilent Microarray Layout and Orientation" on page 63 for appropriate slide layout and orientation in GenePix scanner.

NOTE

Agilent 1x244K, 2x105K, 4x44K and 8x15K CGH microarrays require 5 µm scan resolution, which is only supported in GenePix 4000B.

Step 6. Data Extraction using Feature Extraction Software

The Feature Extraction (FE) software v9.5 supports extraction of microarray TIFF images (.tif) of Agilent CGH microarrays scanned on Agilent Scanner and GenePix 4000B Scanner.

Figure 2 shows an example of Agilent 244K CGH microarray image opened in Feature Extraction software v9.5 in both full and zoomed view.

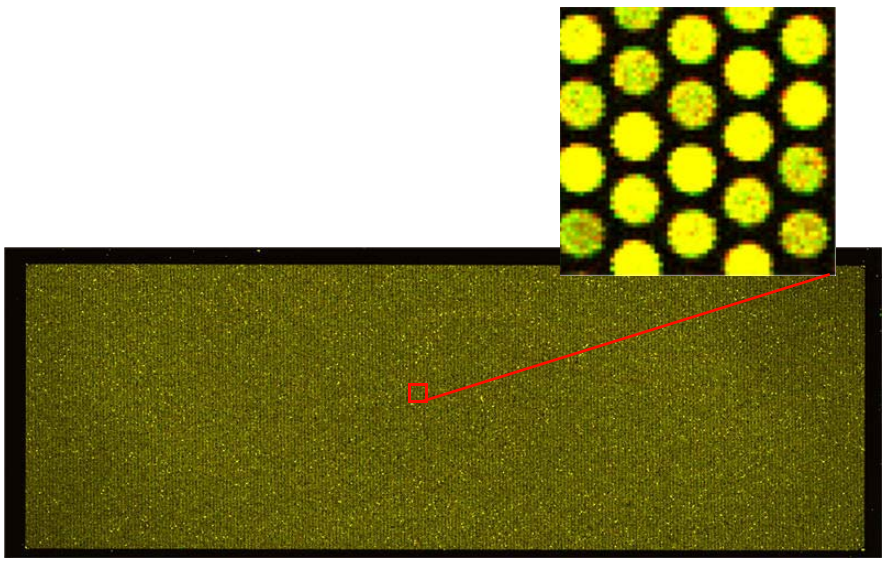


Figure 2 Agilent 244K CGH microarray shown in red and green channels: full and zoomed view

1 Open the Agilent Feature Extraction (FE) program version 9.5.

2 Add the images (.tif) to be extracted to the FE Project.

   a Click **Add New Extraction Set(s)** icon on the toolbar or right-click the Project Explorer and select **Add Extraction...**

   b Browse to the location of the .tif files, select the .tif file(s) and click **Open**. To select multiple files, use the **Shift** or **Ctrl** key when selecting.

   The FE program automatically assigns a default grid template and protocol for each extraction set, if the following conditions are met:

- For auto assignment of the grid template, the image must be generated from an Agilent scanner or GenePix 4000B Scanner and have an Agilent barcode.
- For auto assignment of the CGH FE protocol, the **default CGH protocol** must be specified in the FE Grid Template properties.

  To access the FE Grid Template properties, double-click on the grid template in the Grid Template Browser.

3 Set FE Project Properties.

a Select the **Project Properties** tab.

b In the **General** section, enter your name in the Operator field.

c In all other sections, verify that at least the following default settings as shown in Figure 3 below are selected.

d In the **Other** section, select **CGH_QCM_Feb07**.

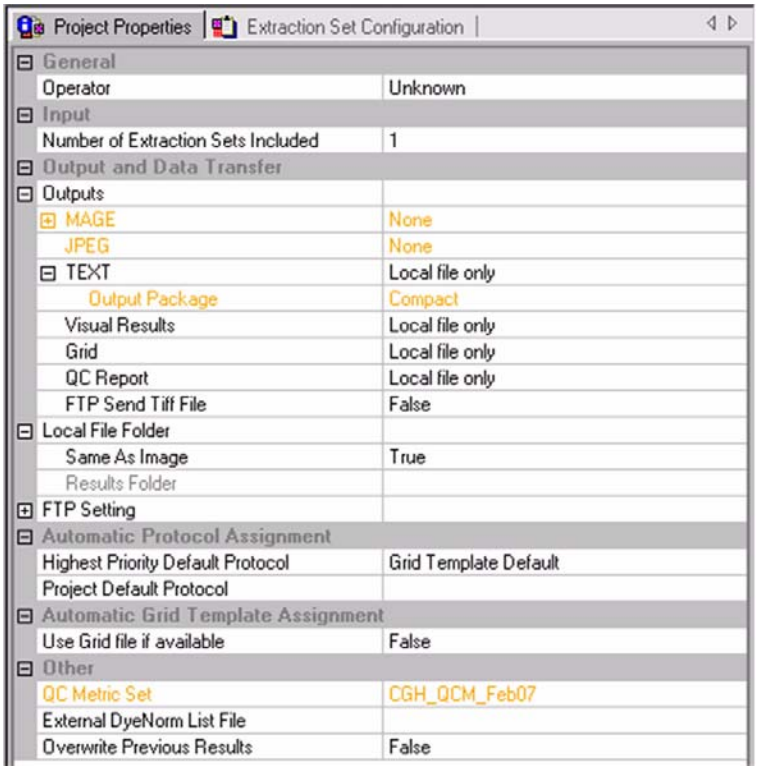


Figure 3 Default settings

4 Check the Extraction Set Configuration.

   a Select the **Extraction Set Configuration** tab.

   b Verify that the correct grid template is assigned to each extraction set in the **Grid Name** column. To assign a different grid template to an extraction set, select one from the pull down menu.

   If a grid template is not available to select from the pull down menu, you must add it to the Grid Template Browser. To add, right-click inside the Grid Template Browser, select **Add**. Look for the design file (.xml) and click **Open** to load grid template into the FE database.

To update to the latest grid templates via Online Update, right-click **Grid Template Browser** and select **Online Update**. You can also download the latest grid templates from Agilent Web site at www.agilent.com/chem/downloaddesignfiles. After downloading, you must add the grid templates to the Grid Template Browser.

After a new grid template is added to the Grid Template Browser, remember to specify the default protocol for the new grid template if you want the Feature Extraction program to automatically assign an FE protocol to an extraction set.

c Verify that the CGH-v4_95 protocol is assigned to each extraction set in the **Protocol Name** column. CGH-v4_95 protocol is designed for Agilent CGH microarrays that were processed using version 5.0 protocol, *Oligonucleotide Array-Based CGH for Genomic DNA Analysis (for FFPE Samples)*.

If a protocol is not available to select from the pull down menu, you must import it to the FE Protocol Browser. To import, right-click the **FE Protocol Browser**, select **Import**. Browse for the FE protocol (.xml) and click **Open** to load the protocol into the FE database. Visit Agilent Web site at www.agilent.com/chem/feprotocols to download the latest protocols.

5 Save the FE Project (.fep) by selecting **File > Save As** and browse for desired location.

6 Verify that the icons for the image files in the FE Project Window no longer have a red X through them. A red X through the icon indicates that an extraction protocol was not selected. If needed, reselect the extraction protocol for that image file.

7 Select **Project > Start Extracting**.

8 After the extraction is completed successfully, view the QC report for each extraction set by double-clicking the QC Report link in the **Summary Report** tab. Determine whether the grid has been properly placed by inspecting **Spot Finding of the Four Corners of the Array**. See Figure 4 and Figure 5.

Refer to the application note *Use of Agilent Feature Extraction Software (v8.1) QC Report to Evaluate Microarray Performance* (publication 5989-3056EN) for more details on quality assessment and troubleshooting with the Feature Extraction QC Report. This technical note can be downloaded from the Agilent Web site at www.agilent.com/chem/dnaapplications.

Microarray QC Metrics for FFPE samples

These metrics are only appropriate for FFPE samples analyzed with Agilent CGH microarrays by following the standard operational procedures provided in this user guide. These metrics are exported to a table in the Feature Extraction QC report (select **CGH_QCM_Feb07** in Project Properties before extraction) and in CGH Analytics. They can be used to assess the relative data quality from a set of microarrays in an experiment. In some cases, they can indicate potential processing errors that have occurred or suggest that the data from particular microarrays might be compromised. Many factors can influence the range of these metrics including the biological sample source, quality of starting gDNA, experimental processing, scanner sensitivity and image processing. The value guidelines presented below represent the thresholds that Agilent has observed when analyzing FFPE samples using version 5.0 protocol, Oligonucleotide Array-Based CGH for Genomic DNA Analysis (for FFPE samples).

BGNoise	<15
Signal Intensity	>100
Signal to Noise	>10
Reproducibility	<0.2
DLRSpread	<0.4

QC Chart Tool

At times the Feature Extraction program is used in a production environment, where the biological samples are similar, microarray processing protocols are identical and monitoring run-to-run consistency is an important goal. The Feature Extraction program can help monitor this consistency with the optional QC Chart Tool. The QC Chart Tool extracts summary statistics from a set of Feature Extraction output text files and can be used to generate metric sets that can be imported into the Feature Extraction program for analysis of each batch of microarrays processed. Only one metric set can be assigned to a Feature Extraction project. When that project is run, the Feature Extraction program summarizes the metric statistics on each microarray's QC report and shows if the thresholds (if any) were exceeded. In addition, at the end of the project, a summary chart can be opened to display graphically what the results are for each metric for each microarray. The QC Chart Tool can be downloaded at www.agilent.com/chem/FEQCMetrics.

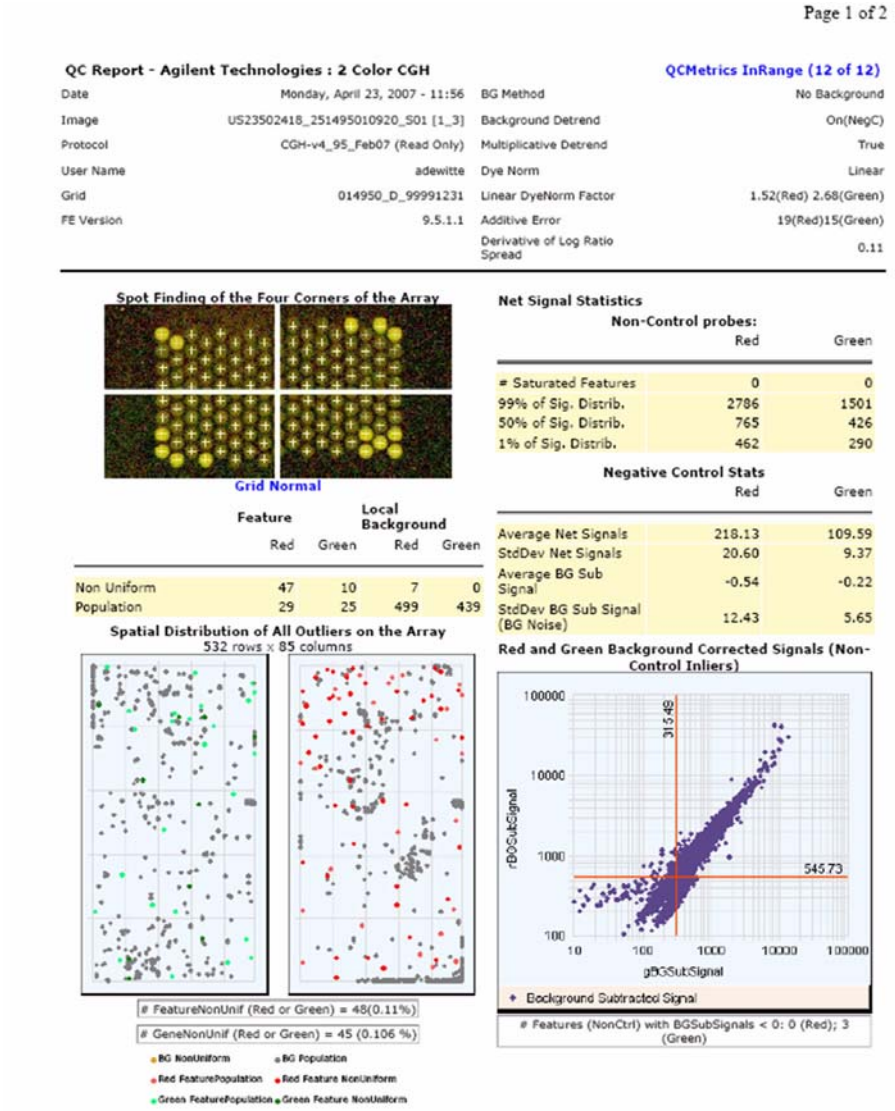
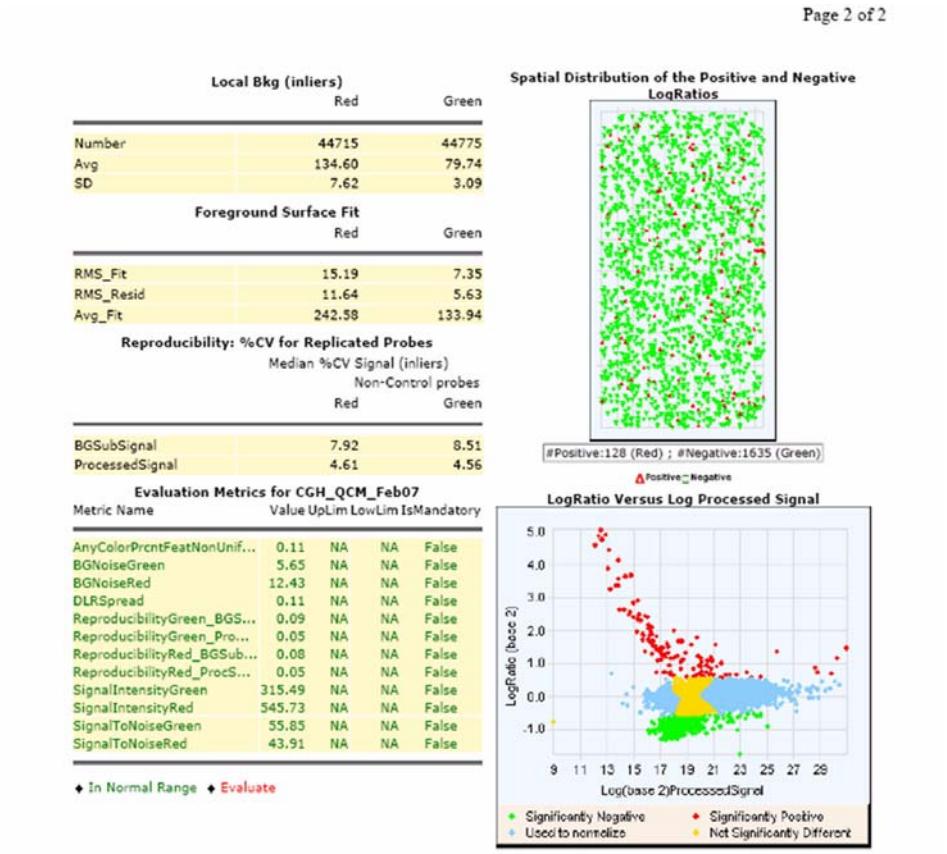


Figure 4 CGH QC report generated from Feature Extraction software v9.5, page 1



Page 2 of 2

Local Bkg (inliers)

	Red	Green
Number	44715	44775
Avg	134.60	79.74
SD	7.62	3.09

Foreground Surface Fit

	Red	Green
RMS_Fit	15.19	7.35
RMS_Resid	11.64	5.63
Avg_Fit	242.58	133.94

Reproducibility: %CV for Replicated Probes

Median %CV Signal (inliers)
Non-Control probes

	Red	Green
BGSubSignal	7.92	8.51
ProcessedSignal	4.61	4.56

Evaluation Metrics for CGH_QCM_Feb07

Metric Name	Value	UpLim	LowLim	IsMandatory
AnyColorPrcntFeatNonUnif...	0.11	NA	NA	False
BGNoiseGreen	5.65	NA	NA	False
BGNoiseRed	12.43	NA	NA	False
DLRSpread	0.11	NA	NA	False
ReproducibilityGreen_BGS...	0.09	NA	NA	False
ReproducibilityGreen_Pro...	0.05	NA	NA	False
ReproducibilityRed_BGSub...	0.08	NA	NA	False
ReproducibilityRed_ProcS...	0.05	NA	NA	False
SignalIntensityGreen	315.49	NA	NA	False
SignalIntensityRed	545.73	NA	NA	False
SignalToNoiseGreen	55.85	NA	NA	False
SignalToNoiseRed	43.91	NA	NA	False

♦ In Normal Range ♦ Evaluate

Spatial Distribution of the Positive and Negative LogRatios

#Positive:128 (Red) ; #Negative:1635 (Green)

LogRatio Versus Log Processed Signal

LogRatio (base 2)
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
9 11 13 15 17 19 21 23 25 27 29
Log(base 2)ProcessedSignal
Significantly Negative
Used to normalize
Significantly Positive
Not Significantly Different

Figure 5 CGH QC report generated from Feature Extraction software v9.5, page 2

5 Reference

Supporting User Guides 62
Agilent Microarray Layout and Orientation 63
Agilent Information Assets Access Agreement 66

This chapter contains reference information that pertains to this protocol.

Supporting User Guides

If you are a first-time user of Agilent's oligo microarray system, please refer to the following user guides for detailed descriptions and operation recommendations for each of the hardware and software components used in the Oligo aCGH application workflow.

The user guides can be downloaded from the Agilent Web site at www.agilent.com/chem/dnamanuals-protocols.

- Agilent Microarray Hybridization Chamber User Guide (p/n G2534-90001)
- Agilent G2545A Hybridization Oven User Manual (p/n G2545-80001)
- Agilent G2565AA and G2565BA Microarray Scanner System User Manual (G2566-90017)
- Agilent Microarray Format Technical Drawings with Tolerances (p/n G4502-90001)
- Agilent Feature Extraction Software Quick Start Guide
- Agilent Feature Extraction Software User Guide
- Agilent Feature Extraction Software Reference Guide

Agilent Microarray Layout and Orientation

Agilent oligo microarray (1 microarray/slide format) as imaged on the Agilent microarray scanner (G2565BA)

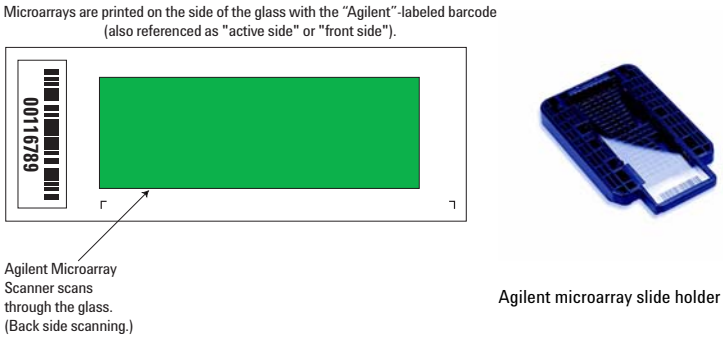

Microarrays are printed on the side of the glass with the "Agilent"-labeled barcode
(also referenced as "active side" or "front side").
00116789
Agilent Microarray
Scanner scans
through the glass.
(Back side scanning.)
Agilent microarray slide holder

Figure 6 Agilent microarray slide and slide holder

Agilent oligo microarray formats and the resulting "microarray design files" are based on how the Agilent microarray scanner images 1-inch × 3-inch glass slides. Agilent designed its microarray scanner to scan through the glass slide (back side scanning). The glass slide is securely placed in an Agilent microarray slide holder with the "Agilent"-labeled barcode facing upside down. In this orientation, the "active side" containing the microarray is protected from potential damage by fingerprints and other elements. Once securely placed, the numeric barcode, "non-active side" of the slide is visible.

Figure 6 depicts how the Agilent microarray scanner reads the microarrays and how this relates to the "microarray design files" that Agilent generates during the manufacturing process of its *in situ*-synthesized oligonucleotide microarrays. Thus, if you have a scanner that reads microarrays from the "front side" of the glass slide, the collection of microarray data points will be different in relation to the "microarray design files" supplied with the Agilent oligo microarray kit you purchased. Therefore, please take a moment to become familiar with the microarray layouts for each of the Agilent oligo microarrays and the layout information as it pertains to scanning using a "front side" scanner.

Non-Agilent Front Side Microarray Scanners

When scanning Agilent oligo microarray slides, the user must determine:

- If the scanner images the microarrays by reading them on the “front side” of the glass slide (“Agilent”-labeled barcode side of the slide) and
- If the microarray image produced by the non-Agilent scanner is oriented in a “portrait” or “landscape” mode, and “Agilent”-labeled barcode is on the left-side, right-side, up or down, as viewed as an image in the imaging software (see Figure 7).

This changes the feature numbering and location as it relates to the “microarray design files” found on the CD in each Agilent oligo microarray kit.

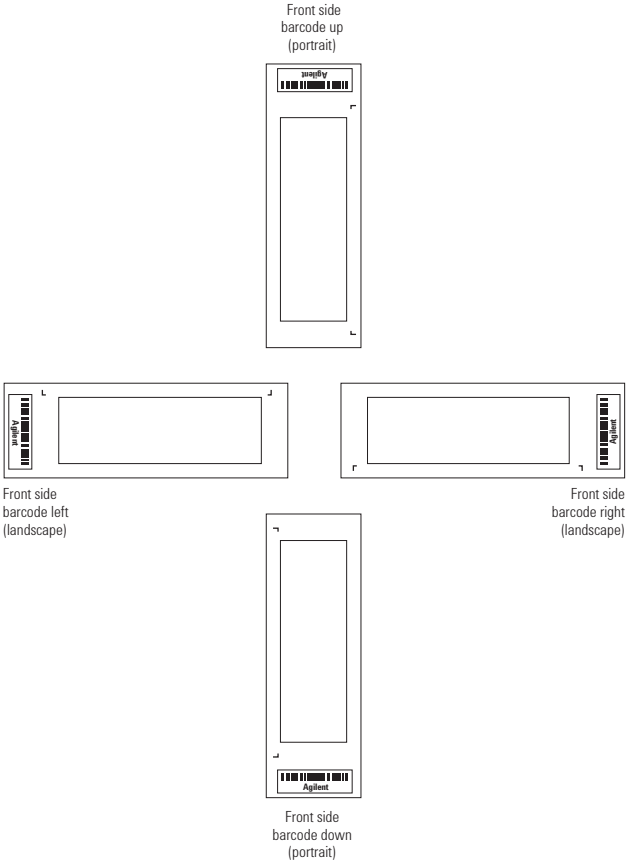


Figure 7 Microarray slide orientation

Agilent Information Assets Access Agreement

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1 Agilent Technologies (“Agilent”) and User have entered into a business relationship that would be fostered by granting User access to certain of Agilent's Information Assets (“Assets”). Accordingly, User and Agilent have entered into this Information Assets Access Agreement (IAAA) in order to protect the confidentiality, integrity, availability, and physical security of Agilent 's various information assets and to prevent unauthorized individuals from gaining access to Agilent's various networks, computers and information assets. Access is granted only for the purposes of fulfilling the specific business relationship between User and Agilent which gives rise to this agreement. The use of Agilent information for any other purpose is expressly prohibited.

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2 This Agreement constitutes the entire and exclusive agreement of the parties regarding the subject matter of this Agreement. This Agreement may not be varied.

5 Reference

Agilent Information Assets Access Agreement

www.agilent.com

In This Book

This guide contains information to run the Oligonucleotide Array-Based CGH for Genomic DNA Analysis (for FFPE Samples) protocol.

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